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Extracellular Hemicellulolytic Enzymes from the Maize Endophyte *Acremonium zeae*

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Abstract Microorganisms that colonize plants require a number of hydrolytic enzymes to help degrade the cell wall. The maize endophyte *Acremonium zeae* was surveyed for production of extracellular enzymes that hydrolyze cellulose and hemicellulose. The most prominent enzyme activity in cell-free culture medium from *A. zeae* NRRL 6415 was xylanase, with a specific activity of 60 U/mg from cultures grown on crude corn fiber. Zymogram analysis following SDS-PAGE indicated six functional xylanase polypeptides of the following masses: 51, 44, 34, 29, 23, and 20 kDa. Xylosidase (0.39 U/mg), arabinofuranosidase (1.2 U/mg), endoglucanase (2.3 U/mg), cellobiohydrolase (1.3 U/mg), and β-glucosidase (0.85 U/mg) activities were also detected. Although apparently possessing a full complement of hemicellulolytic activities, cell-free culture supernatants prepared from *A. zeae* required an exogenously added xylosidase to release more than 90% of the xylose and 80% of the arabinose from corn cob and wheat arabinoxylans. The hydrolytic enzymes from *A. zeae* may be suitable for application in the bioconversion of lignocellulosic biomass into fermentable sugars.

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Introduction

Acremonium zeae Gams & Sumner, one of the most prevalent fungal colonists of preharvest corn, produces symptomless infections of corn seeds and has been isolated from the stalks of mature plants [3, 8, 11, 16]. *A. zeae* has been the subject of recent investigations because of its production of pyrrocidine antibiotics and its potential to serve as a biocontrol agent against mycotoxin-producing fungi [18]. The observation by Harris [5] that, when cultured on artificial media, *A. zeae* "grew most vigorously on medium containing xylan isolated from maize cobs" suggested that *A. zeae* might be a source of hemicellulolytic enzymes uniquely adapted for the utilization of maize cell wall components. *A. zeae* was also found to grow well on the pentose sugars xylose and arabinose and on oat spelt xylan [5; D.T.W., unpublished data], further evidence that it would be a good source of hemicellulolytic enzymes.

Corn fiber is a mixture of corn hulls and residual starch produced during the wet-milling process. It contains about 70% carbohydrate, of which approximately 14% and 35% are in the form of cellulose and hemicellulose, respectively [4]. Its relatively low commercial value, high carbohydrate content, and abundant availability at the wet mills make it an attractive feedstock for fermentation. Recalcitrance of corn fiber hemicellulose to enzymatic hydrolysis, however, poses a significant technical barrier to the commercial use of this feedstock [12]. In the present study, we examined the maize endophtye *A. zeae* strain NRRL 6415 for the production of hydrolytic enzymes when grown on various lignocellulosic carbon sources. Our objective was to determine if enzyme mixtures from this strain are effective at hydrolyzing lignocellulosic biomass, including corn fiber.

Materials and Methods

Preparation of Cell-Free Culture Supernatants

Acremonium zeae NRRL 6415 was obtained from the ARS Culture Collection maintained at the USDA-ARS National Center for Agricultural Utilization Research, Peoria, Illinois. Culture medium contained the following per liter: 2.8 g $NaNO_3$, 2.0 g KH_2PO_4, 1.0 g $MgSO_4 \cdot 7H_2O$, 0.01 g $FeSO_4 \cdot 7H_2O$, and 1% (w/v) carbon source (crude corn fiber [CCF], alkaline hydrogen peroxide-treated corn fiber [AHP-CF], or oat spelt xylan [OSX]). AHP-CF was produced as described by Leathers and Gupta [9]. Cultures were grown at 25°C with shaking at 130 rpm. Cultures were cleared of cells by centrifugation and the supernatant liquid was retained as the cell-free culture supernatant. Protein was determined using the Bio-Rad Protein Assay kit (Bio-Rad Laboratories, Inc., Hercules, CA) against a bovine serum albumin standard.

Assay of Enzymatic Activity

Enzyme assays for polysaccharide and *p*-nitrophenyl substrates were performed as described previously [2]. For polysaccharide substrates, 1 unit (U) of activity is defined as the amount of enzyme that produces 1 μmol of reducing sugar (glucose equivalent) per minute. For *p*-nitrophenyl substrates, 1 U of activity is defined as the amount of enzyme that produces 1 μmol of *p*-nitrophenol per minute. Data for the calculation of kinetic constants were collected under standard assays conditions except for the variation of substrate concentration. K_m and $V_{\max}$ were determined by evaluation of Lineweaver-Burke plots of initial velocity versus substrate concentration.

Temperature optima for xylanase, arabinofuranosidase, and xylosidase activities were determined by varying the assay temperature between 37°C and 85°C under otherwise standard assay conditions. The following discontinuous buffer system was used to determine pH optima for enzyme activities at 37°C: 50 mM sodium acetate (pH 3.5–5.5), 50 mM sodium phosphate (pH 6.0–7.0), 50 mM glycine (8.0–10.0). Temperature stability was determined by incubating cell-free culture supernatants at varying temperatures (37° to 85°C) for 60 min, then measuring residual activity under standard assay conditions.

Zymogram Analysis

Zymogram analysis was performed in 10% SDS-PAGE gels containing 0.1% (w/v) oat spelt xylan polymerized within the gel matrix. Activity was detected as described previously [2].

Hydrolysis of Arabinoxylans

Recombinant *β*-D-xylosidase (SXA) from *Selenomonas ruminantium* was produced as described previously [7]. Reactions (1 ml) contained 40 μg of xylan substrate, 360 μg of SXA, and 5 μg of cell-free culture supernatant in 100 mM sodium succinate, pH 5.3. After 14 h at 25°C, a second dose of cellulase was added, and the mixture incubated for an additional 6 h. Products were analyzed by HPLC as described previously [7].

Results

Hydrolytic Enzyme Activities of *Acremonium zeae* NRRL 6415

A. zeae NRRL 6415 cultures grown on CCF produced 98 ± 10 μg protein/ml in the cell-free culture supernatant, AHP-CF cultures produced 68 ± 7.0 μg protein/ml, and OSX cultures produced 44 ± 1.0 μg protein/ml. Table 1 lists the specific activities of hydrolytic enzymes present in the extracellular matrix. The most prominent activity was xylanase, with a specific activity of 60 U/mg from cultures grown on CCF. Other hemicellulolytic activities detected included xylosidase (pNP-X substrate) and arabinofuranosidase (pNP-A substrate). Cellulolytic activity against

Table 1 Enzyme activities in cell-free culture supernatants of *Acremonium zeae* grown on various carbon sources

Carbon source	Specific activity (U/mg protein)						
	OSX	pNP-X	pNP-A	CMC	pNP-C	pNP-G	Avicel
Crude corn fiber	59–62	0.31–0.47	0.9–1.5	1.9–2.7	1.3–1.3	0.74–0.95	0.8–1.0
AHP-corn fiber	19–24	0.14–0.20	1.0–1.4	1.4–2.0	1.5–1.7	0.68–0.77	0.9–1.9
Oatspelt xylan	33–39	0.29–0.45	1.2–1.6	0.80–1.2	1.9–2.4	0.48–0.49	1.4–2.2

Note: Enzyme activities were measured in cell-free culture supernatants from 14-day cultures. Substrates included the following: OSX, oatspelt xylan; pNP-X, 4-nitrophenyl *β*-D-xylopyranoside; pNP-A, 4-nitrophenyl *α*-L-arabinofuranoside; CMC, carboxymethylcellulose; pNP-C, 4-nitrophenyl *β*-D-cellobioside; and pNP-G, 4-nitrophenyl *β*-D-glucopyranoside. Standard assay conditions were pH 5.0 at 37°C. One unit (U) of activity is defined as the amount of enzyme that produces 1 μmol of product (reducing sugar or *p*-nitrophenol) per minute. Data are reported as the range of activity from two independent cultures

CMC (representing endoglucanase), pNP-C and avicel (representing cellobiohydrolase), and pNP-G (β-glucosidase) was also detected. No activity against soluble starch (amylase) was detected.

Zymogram analysis indicated that the CCF culture possessed at least six active polypeptides of 51, 44, 34, 29, 23, and 20 kDa (Fig. 1). The OSX culture displayed four active polypeptides (51, 34, 23, and 20 kDa), and the AHP-CF culture only three (51, 34, and 20 kDa).

The pH and temperature for optimum xylanase activity in cultures grown on crude corn fiber were pH 5.5 and 65°C. The enzyme had a broad pH range, retaining more than 37% of its maximal activity at 65°C over the pH range from 4.5 to 9.0. At pH 5.5, it retained 68% of maximal activity at 75°C, but enzyme activity was not stable at higher temperatures, losing 23%, 65%, and 98% of its activity following 60 min of incubation at 45, 55, and 65°C, respectively. Temperature and pH optima for the pNP-arabinofuranosidase activity were 70°C and pH 5.0, and the optima for the pNP-xylosidase activity were 65°C and pH 5.5. Kinetic parameters for xylanase, pNP-arabinofuranosidase, and pNP-xylosidase activities from cultures grown on crude corn fiber, AHP-treated corn fiber, and oat spelt xylan are listed in Table 2.

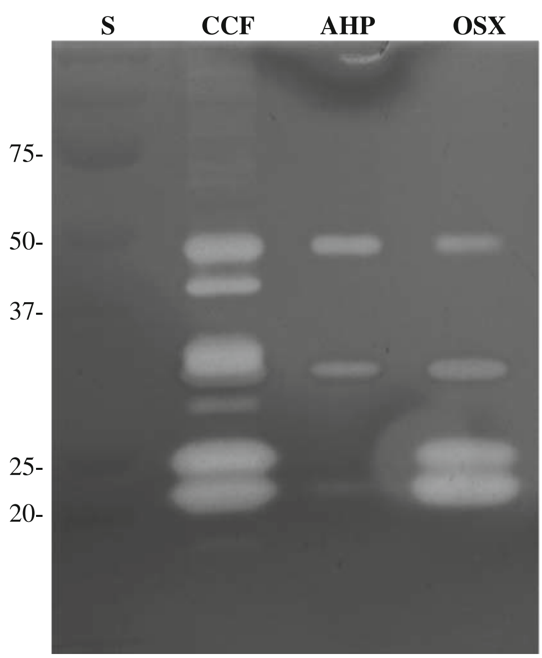

Fig. 1 Zymogram analysis for xylanase activity. Each lane contains 0.5 µg of cell-free culture supernatant. Lane designations are as follows: S, Bio-Rad Precision Plus protein standards; CCF, culture grown on crude corn fiber; AHP, culture grown on AHP-treated corn fiber; OSX, culture grown on oat spelt xylan. The masses of molecular markers (kDa) are listed at the left

Hydrolysis of Purified Arabinoxylans

Analysis of the hydrolysis products of wheat arabinoxylan indicated that xylobiose accumulated in the reaction in addition to the monosaccharides arabinose and xylose (data not shown). Addition of an exogenous recombinant xylosidase (SXA) converted the accumulated xylobiose to xylose. Table 3 lists the percentages of sugars released from isolated arabinoxylans by *A. zeae* cell-free culture supernatant supplemented with SXA.

Discussion

The heterogeneous structure of hemicellulose requires a complex set of enzymatic activities for complete hydrolysis [13]. Endophytes represent a rich and diverse source of xylanase producing microorganisms [17], and as one of the most prevalent colonists of preharvest corn, *A. zeae* may be a source of enzymes uniquely suited for utilization of the corn cell wall. Although other species of *Acremonium* have previously been studied for production of cellulolytic enzymes [6, 20], there are few data available on the production of hemicellulases. A fungal culture (strain Y-94) identified as '*Acremonium cellulolyticus*' nom. inval. [21] had been shown to produce large amounts of cellulolytic enzymes but only 3.4 U/mg xylanase [10, 20]. In the present study, *A. zeae* NRRL 6415 produces more xylanolytic than cellulolytic activity, but with a specific activity of xylanase almost 20-fold higher than that reported for *A.*

Table 2 Kinetic parameters for enzymatic activities in *A. zeae* cell-free culture supernatants

Substrate	Crude corn fiber culture		AHP-corn fiber culture		Oat spelt xylan culture	
	K_m	V_{max} (U/mg)	K_m	V_{max} (U/mg)	K_m	V_{max} (U/mg)
Oat spelt xylan	5.7 ± 0.4 mg/ml	62 ± 2.5	4.0 ± 0.3 mg/ml	43 ± 2.3	6.7 ± 0.3 mg/ml	74 ± 3.0
pNP-A	0.55 ± 0.02 mM	1.9 ± 0.1	0.52 ± 0.02 mM	1.3 ± 0.1	0.67 ± 0.03 mM	0.84 ± 0.03
pNP-X	0.95 ± 0.11 mM	0.47 ± 0.03	1.0 ± 0.1 mM	0.26 ± 0.01	0.77 ± 0.09 mM	0.27 ± 0.02

Note: pNP-A, 4-nitrophenyl α-L-arabinofuranoside; pNP-X, 4-nitrophenyl β-D-xylopyranoside. Kinetic parameters were determined in cell-free culture supernatants from 3-day cultures of *A. zeae* grown on either crude corn fiber, AHP-treated corn fiber, or oat spelt xylan. Values are reported as the mean ± standard deviation from three experiments

Table 3 Hydrolysis of isolated arabinoxylans to monosaccharides

Substrate	CCF culture		AHP-CF culture		OSX culture		SXA	
	L-Ara	D-Xyl	L-Ara	D-Xyl	L-Ara	D-Xyl	L-Ara	D-xyl
Corn cob xylan	82	101	74	96	67	90	0.2	13
Oat spelt xylan	62	70	54	62	54	58	0.4	7
Wheat arabinoxylan								
Low viscosity	82	89	80	85	80	85	0.1	7
High viscosity	96	104	89	97	90	99	0.2	3
Beechwood xyl	–	87	–	80	–	82	–	22

Note: Ara, arabinose; xyl, xylose. Values are reported as the mole percentage of monosaccharide detected following enzymatic hydrolysis of each substrate relative to that for hydrolysis with TFA. Conditions for hydrolysis of arabinoxylans are described under Materials and Methods. Complete hydrolysis of each substrate with 2 N trifluoroacetic acid yielded the following concentrations (μM L-arabinose, μM D-xylose): corn cob xylan (31, 142), oat spelt xylan (24, 199), low-viscosity wheat arabinoxylan (61, 171), high-viscosity wheat arabinoxylan (85, 160), and beechwood xylan (0, 193)

cellulolyticus strain Y-94. It should be noted, however, that Y-94 produced over 70-fold more protein in culture broth than *A. zeae* NRRL 6415. Improvement of the growth medium enhanced production by Y-94 approximately 10-fold [20], and thus, significant increases in *A. zeae* enzyme yield could be achieved by optimizing the culture conditions.

Zymogram analysis suggests that *A. zeae* produces multiple xylanases. Consistent with the relative amounts of xylanase activity, six active polypeptides were observed in cultures grown on crude corn fiber, compared with four in cultures grown on oat spelt xylan and only three in cultures grown on AHP-treated corn fiber. Production of multiple xylanases is a common occurrence in microorganisms and may reflect the need for a suite of enzymes with different substrate specificities to efficiently hydrolyze xylan [1, 10, 19]. From the present data, one cannot conclude whether the different polypeptides are the products of distinct genes or artifacts of posttranslational modifications (such as glycosylation or proteolysis) of a single enzyme. To date, there are no genome data available for any *Acremonium* species, and therefore further genetic analysis is warranted to determine the number and expression levels of each *Acremonium* xylanase.

A number of other extracellular hydrolytic enzyme activities were produced by *A. zeae*. Although xylosidase activity is detected in the *A. zeae* preparations, there may not be sufficient activity, or the *A. zeae* enzyme might not have the proper substrate specificity, to fully hydrolyze the wheat arabinoxylans. Thus, an exogenous xylosidase was needed to hydrolyze the substrate to monomeric sugars. This is consistent with the reported synergism between endo-1,4-xylanases and α-L-arabinofuranosidases from commercial cellulases when used to hydrolyze wheat arabinoxylan [14, 15]. While Sorensen and colleagues previously reported yields of arabinose and xylose from wheat arabinoxylan of 46% and 89%, respectively [14], it should be noted that these numbers are saturation yields, resulting from hydrolysis of at least 2.5 wt% dry matter. In the present study, the use of a low concentration of arabinoxylan was intended to avoid product inhibition of the enzymes and mimic the low product concentrations likely to be encountered in a simultaneous saccharification and fermentation process. Direct comparison of the *A. zeae* activities to those reported previously is therefore difficult, but the data suggest that *A. zeae* enzymes may prove suitable for application in a simultaneous saccharification and fermentation process.

In conclusion, evidence is offered that the maize endophyte *Acremonium zeae* NRRL 6415 may be uniquely adapted for the utilization of maize cell wall components. It produces a full complement of hemicellulolytic enzymes capable of hydrolysis of arabinoxylans from several industrially important feedstocks. Further investigation of the production and properties of *A. zeae* hemicellulases is warranted to facilitate development of enzymes to convert agricultural biomass into fuels and chemicals.

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